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DIFFERENTIAL TURNOVER OF ISOFLAVONE 7-*O*-GLUCOSIDE-6″-*O*-MALONATES IN *CICER ARIETINUM* ROOTS

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Key Word Index—*Cicer arietinum*, Leguminosae, isoflavone 7-*O*-glucoside-6″-*O*-malonate, turnover, L-α-amino-oxy-β-phenylpropionic acid, formononetin

Abstract—Pulse labelling experiments and studies with a molecular inhibitor of phenylalanine ammonia lyase showed that in roots of *Cicer arietinum* formononetin 7-*O*-glucoside-6″-*O*-malonate is rapidly metabolized whereas biochanin A 7-*O*-glucoside-6″-*O*-malonate appears to be metabolically rather inert

INTRODUCTION

In recent years several investigations have demonstrated the increasing importance of *O*- and *N*-malonyl compounds in plant metabolism Thus, malonyl conjugates of flavone, flavonol, anthocyanidin and isoflavone glucosides [1–6], of D-configurated amino acids [7, 8], of intermediates or end products of pesticide degradation in plants [9, 10], of riboflavin as the possible blue light photo-receptor in *Avena* coleoptiles [11], and finally of the ethylene precursor 1-amino-1-carboxycyclopropane [12–14] have been isolated The *N*-malonyl-1-amino-1-carboxycyclopropane and other *N*-malonyl conjugates of xenobiotics appear to be metabolically stable end products [14–17] In parsley cell cultures, the various flavone and flavonol glucoside malonates must also be considered as products with very little turnover [18] The metabolic activity of malonate esters must thus be considered an open question

In *Cicer arietinum* L roots, the isoflavone formononetin (7-hydroxy-4′-methoxyisoflavone) is subject to rapid turnover [18–20], whereas the higher homologue biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) is only very slowly metabolized These early measurements of isoflavone turnover considered the total pools of isoflavones because complete hydrolyses of conjugates were carried out prior to isoflavone determinations Different rates of turnover for aglycone and conjugates can therefore not be excluded

Our recent investigations [5] have shown that in *C arietinum* roots the isoflavone 7-*O*-glucoside-6″-*O*-malonates constitute some 85–90% of the pools of formononetin and biochanin A each, with the rest being divided between the aglycones and the isoflavone 7-*O*-glucosides, respectively We have now used this system to determine separately the turnover of isoflavone aglycones, glucosides, and, most important, the malonate esters Furthermore, it was of interest to examine whether inhibition of isoflavone biosynthesis would lead to an increased level of aglycones and/or glucosides caused by preferential efflux of isoflavone material from the pools of the malonate conjugates

RESULTS AND DISCUSSION

The turnovers of formononetin and biochanin A and their conjugates were measured by two independent methods First, we applied to roots of *C arietinum* L-α-aminooxy-β-phenylpropionic acid (AOPP) as an inhibitor of phenylalanine ammonia lyase [20] and subsequently measured the quantitative changes in the levels of aglycones and isoflavone conjugates Such measurements were conducted with suitable extracts of root tissue by our well-established HPLC techniques [5, 21] Second, pulse labelling studies with [side chain-3-^{14}C]cinnamic acid and L-[1-^{14}C]phenylalanine were also conducted [22] The labelled compounds obtained during these studies were also separated and quantitated by HPLC and their radioactivity was determined by scintillation methods

Application of AOPP implies that the formation of the phenylalanine-derived isoflavones [23] is prevented, whereas further metabolism (turnover) should proceed unaltered The data in Fig 1 (A and D) show that the accumulation of both formononetin and biochanin A 7-*O*-glucoside-6″-*O*-malonate is stopped after 24 hr of incubation with the inhibitor In the following more than 300 hr, the level of biochanin A 7-*O*-glucoside-6″-*O*-malonate in the roots hardly changed indicating that there was practically no turnover of this compound The same results were found both with the aglycone and the glucoside pool of this isoflavone (Fig 1, E and F)

In contrast to these observations, formononetin 7-*O*-glucoside-6″-*O*-malonate showed a substantial turnover, with a biological half-life of *ca* 140 hr (Fig 1, A) Significant or similar rates of turnover, however, could not be measured at the level of formononetin glucoside or aglycone (Fig 1, B and C) because the amounts isolated from AOPP-treated plants were rather constant over the experimental period

The observed turnover of the malonate conjugate of formononetin is presently best explained by an esterase-catalysed conversion to the glucoside (unpublished observations), with the latter compound being hydrolysed by isoflavone glucoside-specific glucohydrolases [24] to yield

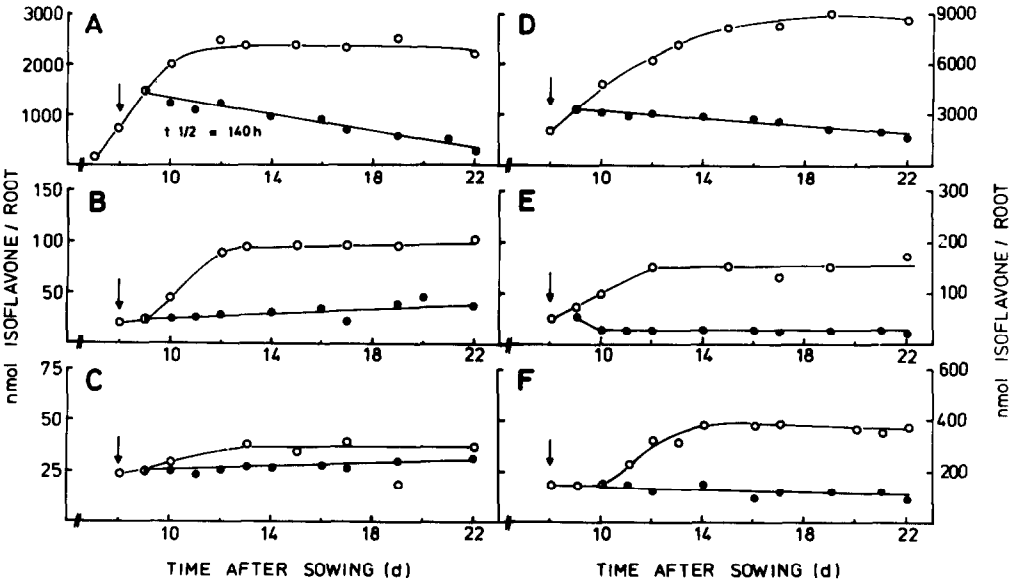

Fig 1 Turnover studies on formononetin (left-hand side) and biochanin A (right-hand side) constituents in *Cicer arietinum* roots with L-α-aminooxy-β-phenylpropionic acid (○——○) Accumulation of isoflavone 7-*O*-glucoside-6″-*O*-malonates (A and D), of isoflavone 7-*O*-glucosides (B and E) and isoflavone aglycones (C and F) in control plants (●——●) Levels of constituents in inhibitor-treated plants The arrow indicates the first application of AOPP

the aglycone Degradative reactions of formononetin itself can also be assumed to occur in *C arietinum* roots [25] This model implies that during the experimental period shown in Fig 1, A–C, substantial amounts of isoflavone material must have passed from the pool of the malonate conjugate through the pools of both formononetin 7-*O*-glucoside and the aglycone Since this flow of isoflavone material did not lead to an equivalent increase in the level of these two pools, as indicated by Fig 1, B and C, it must be assumed that the glucoside and the aglycone are as rapidly metabolized as the malonate conjugate Inspection of the HPLC diagrams of the experiments shown in Fig 1 did not indicate the presence of any new aromatic compound which might be regarded as a conversion or a degradation product of formononetin The pathway of formononetin degradation remains to be elucidated

Two repetitions of the experiments with AOPP resulted in essentially the same values for the biological half-life of formononetin 7-*O*-glucoside-6″-*O*-malonate (110–120 hr) and biochanin A 7-*O*-glucoside-6″-*O*-malonate (> 350 hr), with all the other compounds remaining practically unchanged in the control and the AOPP-treated plants

Pulse labelling experiments were conducted at the same growth period of the plants as shown in Fig 1 The accumulation curves obtained for aglycones and conjugates of the two isoflavones were identical to those obtained in the AOPP experiments (Fig 1) Both L-[1-^{14}C]phenylalanine and [side chain -3-^{14}C]cinnamic acid were used as precursors Previous experiments had shown a substantial difference in the metabolic conversion of these two precursors into the isoflavones of chickpea plants [22] Regardless of the precursor, however, formononetin 7-*O*-glucoside-6″-*O*-malonate showed a rapid turnover ($t_{1/2} \approx$ 140–160 hr), as measured by the decrease in the total radioactivity and the specific radioactivity of the isolated product (data not shown but see ref [22]) In the case of the equivalent biochanin A conjugate, as well as biochanin A 7-*O*-glucoside and aglycone, significant rates of turnover were not observed ($t_{1/2} >$ 320 hr) The radioactivity curves obtained for formononetin 7-*O*-glucoside with [^{14}C]cinnamic acid turned out to be of great interest because they indicated that the turnover of this compound occurred with a half-life of approximately 240–260 hr Such data support the above assumption that the turnover of the malonate conjugate may be explained by hydrolysis to the glucoside and subsequent formation of the aglycone This assumption is in line with the observation that the specific radioactivity of the glucoside was substantially higher than that obtained for formononetin 7-*O*-glucoside-6″-*O*-malonate

In general, the present results clearly support the earlier findings on the differential turnovers of isoflavones in chickpea, with the 5-desoxyisoflavone formononetin being the metabolically active compound [19, 20, 22] Furthermore, the data prove that malonyl glucosides occur in steady-state concentrations and that they may be turned over with significant rates This turnover requires that there is a continuous influx and efflux of such compounds from the vacuolar compartment of the cells [26] Root cells of chickpea plants clearly offer a suitable system for studying the metabolism of the metabolically very active formononetin 7-*O*-glucoside-6″-*O*-malonate at the cellular level Such studies are in progress

EXPERIMENTAL

Plant material Commercially available seeds of *Cicer arietinum* were surface-sterilized with 70% EtOH and germinated under

flowing tap H_2O for 4 days Seedlings were transferred to an incubation chamber (25°, 10 klx, light–dark programme, 16 8 hr) with the roots immersed in H_2O The gas phase of the chamber was flushed constantly with a H_2O-saturated, gentle air stream Experiments were started on the eighth day after sowing The roots of three plants each were worked up for isoflavone determination

Feeding experiments For application of L-α-aminooxy-β-phenylpropionic acid, the roots of the experimental plants were kept in a 0 3 mM soln of the inhibitor for the whole incubation period For studies with L-[1-^{14}C]phenylalanine (sp radioact 57 mCi/mmol, Amersham–Buchler), 80 plants were given 60 μCi of precursor for 10 hr After this period, the roots were washed carefully with H_2O and further incubated under normal conditions For investigations with [side chain-3-^{14}C]cinnamic acid (sp radioact 48 mCi/mmol, Amersham–Buchler), 50 plants were root-fed with 25 μCi dissolved in 0 4% $NaHCO_3$ soln, pH 8 0, for 10 hr Subsequent growth of the plants was as described above $^{14}CO_2$ was absorbed in a mixture of methoxyethanol–ethanolamine (2 1) [22]

Quantitation of isoflavones Extraction of isoflavones and their conjugates from root tissue, preparation of samples for analysis, the HPLC chromatography and concomitant quantitation of compounds were carried out as described previously [5, 21]

For determination of radioactivity of substrates, the plant extracts were lyophilized and the resulting powder was dissolved in minimum amounts of dimethyl sulphoxide Aliquots of these solns were subjected to HPLC separation essentially as described but with the aid of a preparative column (RP 8 Lichrosorb, 7 μm, 250 × 8 mm) and a stepwise gradient of first 20% B to 35% B in (A + B) for 20 min and then from 35% B to 60% B in (A + B) for an additional 20 min Solvent A was 1 5% phosphoric acid and acetonitrile was used as solvent B The HPLC eluate was fractionated (30 sec intervals) and the fractions counted as described [22] Counting efficiency was determined by internal standardization with [^{14}C]toluene

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